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(54) Title: METHOD OF TREATING ATHEROSCLEROSIS OR RESTENOSIS USING MICROTUBULE STABILIZING AGENT

(57) Abstract

The present invention is a method of preventing or reducing atherosclerosis or restenosis, and a pharmaceutical preparation used therefor. In particular, it is a method of preventing or reducing atherosclerosis or restenosis after arterial injury by treatment with a low dose of a microtubule stabilizing agent such as taxol or a water soluble taxol derivative. The low dose used in the present invention prevents artery blockage while minimizing any negative side effects associated with the drug.

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METHOD OF TREATING ATHEROSCLEROSIS OR
RESTENOSIS USING MICROTUBULE STABILIZING AGENT

Field of the Invention

The present invention relates to a method of treating patients at risk of developing atherosclerosis or restenosis.

More particularly, the invention relates to treatment of these patients with a low dose taxol solution to prevent or reduce the development of atherosclerosis or restenosis.

Background of the Invention

Vascular disease is the leading cause of death and disability in the developed world, particularly afflicting the elderly. In the United States alone, despite recent encouraging declines, cardiovascular disease is still responsible for almost one million fatalities each year and more than one half of all deaths; almost 5 million persons afflicted with cardiovascular disease are hospitalized each year. The cost of this disease in terms of human suffering and of material resources is almost incalculable.

Atherosclerosis is the most common form of vascular disease and leads to insufficient blood supply to critical body organs, resulting in heart attack, stroke, and kidney failure. Additionally, atherosclerosis causes major complications in those suffering from hypertension and diabetes, as well as tobacco smokers. Atherosclerosis is a form of chronic vascular injury in which some of the normal vascular smooth muscle cells ("VSMC") in the artery wall, which ordinarily control vascular tone regulating blood flow, change their nature and develop "cancer-like" behavior. These VSMC become abnormally proliferative, secreting substances (growth factors, tissue-degradation enzymes and other proteins) which enable them to invade and spread into the inner vessel lining, blocking blood flow and making that vessel abnormally susceptible to

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being completely blocked by local blood clotting, resulting in the death of the tissue served by that artery.

Restenosis, the recurrence of stenosis or artery stricture after corrective surgery, is an accelerated form of atherosclerosis. Recent evidence has supported a unifying hypothesis of vascular injury in which coronary artery restenosis along with coronary vein graft and cardiac allograft atherosclerosis can be considered to represent a much accelerated form of the same pathogenic process that results in spontaneous atherosclerosis (Ip, J.H., et al., (1990) J Am Coll Cardiol, 15:1667-1687; Muller, D.W.M., et al., (1992) J Am Coll Cardiol, 19:418-432). Restenosis is due to a complex series of fibroproliferative responses to vascular injury involving potent growth-regulatory molecules, including platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), also common to the later stages in atherosclerotic lesions, resulting in vascular smooth muscle cell proliferation, migration and neointimal accumulation.

Restenosis occurs after coronary artery bypass surgery (CAB), endarterectomy, and heart transplantation, and particularly after heart balloon angioplasty, atherectomy, laser ablation or endovascular stenting (in each of which one-third of patients redevelop artery-blockage (restenosis) by 6 months), and is responsible for recurrence of symptoms (or death), often requiring repeat revascularization surgery. Despite over a decade of research and significant improvements in the primary success rate of the various medical and surgical treatments of atherosclerotic disease, including angioplasty, bypass grafting and endarterectomy, secondary failure due to late restenosis continues to occur in 30-50% of patients (Ross, R. (1993) Nature, 362:801-809).

As a result, a need exists for a successful

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chemotherapeutic therapy to reduce or prevent artery-
blockage. The most effective way to prevent this disease
is at the cellular level, as opposed to repeated
revascularization surgery which can carry a significant
risk of complications or death, consumes time and money,
and is inconvenient to the patient.

Microtubules, cellular organelles present in all
eukaryotic cells, are required for healthy, normal
cellular activities. They are an essential component of
the mitotic spindle needed for cell division, and are
required for maintaining cell shape and other cellular
activities such as motility, anchorage, transport between
cellular organelles, extracellular secretary processes
(Dustin, P. (1980) Sci. Am., 243: 66-76), as well as
modulating the interactions of growth factors with cell
surface receptors, and intracellular signal transduction.
Furthermore, microtubules play a critical regulatory role
in cell replication as both the c-mos oncogene and CDC-2-
kinase, which regulate entry into mitosis, bind to and
phosphorylate tubulin (Verde, F. et al. (1990) Nature,
343:233-238), and both the product of the tumor suppressor
gene, p53, and the T-antigen of SV-40 bind tubulin in a
ternary complex (Maxwell, S.A. et al. (1991) Cell Growth
Differen., 2:115-127). Microtubules are not static, but
are in dynamic equilibrium with their soluble protein
subunits, the α- and ß-tubulin heterodimers. Assembly
under physiologic conditions requires guanosine
triphosphate (GTP) and certain microtubule associated and
organizing proteins as cofactors; on the other hand, high
calcium and cold temperature cause depolymerization.

Interference with this normal equilibrium
between the microtubule and its subunits would therefore
be expected to disrupt cell division and motility, as well
as other activities dependent on microtubules. This
strategy has been used with significant success in the
treatment of certain malignancies. Indeed,

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antimicrotubule agents such as colchicine and the vinca
alkaloids are among the most important anticancer drugs.
These antimicrotubule agents, which promote microtubule
disassembly, play principal roles in the chemotherapy of
most curable neoplasms, including acute lymphocytic
leukemia, Hodgkin's and non-Hodgkin's Lymphomas, and germ
cell tumors, as well as in the palliative treatment of
many other cancers.

The newest and most promising antimicrotubule
agent under research is taxol. Taxol is an
antimicrotubule agent isolated from the stem bark of _Taxus_
brevifolia, the western (Pacific) yew tree. Unlike other
antimicrotubules such as colchicine and the vinca
alkaloids which promote microtubule disassembly, taxol
acts by promoting the formation of unusually stable
microtubules, inhibiting the normal dynamic reorganization
of the microtubule network required for mitosis and cell
proliferation (Schiff, P.B., et al. (1979) _Nature_ 277:
665; Schiff, P.B., et al. (1981) _Biochemistry_ 20: 3247).
In the presence of taxol, the concentration of tubulin
required for polymerization is significantly lowered;
microtubule assembly occurs without GTP and at low
temperatures, and the microtubules formed are more stable
to depolymerization by dilution, calcium, cold, and
inhibitory drugs. Taxol will reversibly bind to
polymerized tubulin, and other tubulin-binding drugs will
still bind to tubulin even in the presence of taxol.

Taxol has one of the broadest spectrum of
antineoplastic activity, renewing serious interest in
chemotherapeutic strategies directed against microtubules
(Rowinsky, E.K., et al. (1990) _Jrnl. of the Nat'l. Cancer_
Inst., 82:1247-1259). In recent studies, taxol has shown
significant activity in advanced and refractory ovarian
cancer (Einzig, A.I., et al. (1992) _J. Clin. Oncol._,
10:1748), malignant melanoma (Einzig, A.I. (1991) _Invest._
New Drugs, 9:59-64), as well as in cancers of the breast

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(Holmes, F.A., et al. (1991) JNCI, 83:1797-1805), head and
neck, and lung.

        Taxol has been studied for its effect in
combating tumor growth in several clinical trials using a
variety of administration schedules. Severe allergic
reactions have been observed following administration of
taxol. However, it is has been demonstrated that the
incidence and severity of allergic reactions is affected
by the dosage and rate of taxol infusion (Weiss, R.B., et
al. (1990) J. Clin. Oncol. 8: 1263).

        Cardiac arrhythmias are associated with taxol
administration, and like allergic reactions, their
incidence is affected by the dosage and rate of taxol
administration. Sinus bradycardia and Mobitz II
arrhythmia will develop in approximately 40% and 5% of
patients, respectively, beginning 4-6 hours after the
start of a taxol infusion, and continuing for 4-8 hours
after its completion. In most patients, the abnormal
rhythm is transient, asymptomatic, hemodynamically stable,
and does not require cardiac medications or electrical
pacing. Additionally, it has been observed that the
incidence of severe cardiac events is low in patients
receiving taxol alone. Thus, infusion times up to 24
hours have been used in treatment with taxol to decrease
the incidence of toxicity and allergic reaction to the
drug.

        During angioplasty, intraarterial balloon
catheter inflation results in deendothelialization,
disruption of the internal elastic lamina, and injury to
medial smooth muscle cells. While restenosis likely
results from the interdependent actions of the ensuing
inflammation, thrombosis, and smooth muscle cell
accumulation (Ferrell, M., et al. (1992) Circ., 85:1630-
1631), the final common pathway evolves as a result of
medial VSMC dedifferentiation from a contractile to a
secretory phenotype. This involves, principally, VSMC

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secretion of matrix metalloproteinases degrading the
surrounding basement membrane, proliferation and
chemotactic migration into the intima, and secretion of a
large extracellular matrix, forming the neointimal
fibropoliferative lesion. Much of the VSMC phenotypic
dedifferentiation after arterial injury mimics that of
neoplastic cells (i.e., abnormal proliferation, growth-
regulatory molecule and protease secretion, migration and
basement invasion).

     Although others have investigated the use of the
antimicrotubule agent colchicine in preventing restenosis,
opposite conclusions have been reported (See Currier, et
al., "Colchicine Inhibits Restenosis After Iliac
Angioplasty In The Atherosclerotic Rabbit" (1989) Circ.,
80:II-66; O'Keefe, et al., "Ineffectiveness Of Colchicine
For The Prevention Of Restenosis After Coronary
Angioplasty" (1992) J. Am. Coll. Cardiol., 19:1597-1600).
The art fails to suggest the use of a microtubule
stabilizing agent such as taxol in preventing or reducing
this disease. Thus, the method of the present invention
is to prevent or reduce the development of atherosclerosis
or restenosis using a microtubule stabilizing agent such
as taxol or a water soluble taxol derivative. This
microtubule stabilizing mechanism of atherosclerosis or
restenosis prevention is supported by the analogous
results in experiments on cellular proliferation and
migration using taxol and ^2H_2O (deuterium oxide), which
exert comparable microtubule effects via different
underlying mechanisms.

     Accordingly, an object of this invention is to
provide a method to reduce or prevent the development of
atherosclerosis or restenosis using treatment with a drug
which promotes highly stabilized tubule formation.

     An additional object of this invention is to
provide a method of preventing or reducing atherosclerosis
or restenosis using a pharmaceutical preparation

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containing a low dosage of taxol or water soluble taxol
derivative.

All references cited are herein incorporated by
reference.

Summary of the Invention

In accordance with these and other objects of
the present invention, a method of preventing or reducing
atherosclerosis or restenosis is provided, which comprises
treatment with a therapeutically effective amount of a
microtubule stabilizing agent such as taxol or a water
soluble taxol derivative. A therapeutically effective
amount of agent is an amount sufficient to prevent or
reduce the development of atherosclerosis or restenosis.

This method provides an effective way of
preventing or reducing the development of atherosclerosis
or restenosis in those patients susceptible to such
disease. Additionally, because of the low dose of
chemotherapeutic agent used, the chance of a patient
developing adverse reactions is potentially reduced.

BRIEF DESCRIPTION OF FIGURES

FIGURE 1 depicts the taxol induced impairment of
the ability of VSMC to invade filters coated with basement
membrane proteins, and taxol inhibition of cultured VSMC
[^{3}H]-thymidine incorporation.

FIGURE 2 shows taxol inhibition of smooth muscle
cell neointimal accumulation after balloon catheter injury
of the rat carotid artery.

FIGURE 3 depicts deuterium oxide dose-dependent
inhibition of VSMC chemoinvasion, and deuterium oxide
inhibition of cultured VSMC bromodeoxyuridine (BrDU)
incorporation.

FIGURE 4 shows concentrations of taxol caused
dose-dependent microtubule bundling in VSMC's cultured on
plastic.

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FIGURE 5 shows deuterium oxide induced
microtubule bundling in cultured VSCM's.

DETAILED DESCRIPTION OF THE INVENTION

The practice of an embodiment in the present
invention may be accomplished via several alternative drug
delivery routes, such as intraperitoneal or subcutaneous
injection, continuous intravenous infusion, oral ingestion
or local (direct) delivery, or a combination of two or
more. When formulating a solution for injection or
continuous infusion, one must first prepare a taxol
solution. Taxol is supplied through CTEP, DCT, NCI
(IND#22850) as a concentrated solution, 6 mg/ml, in 5 ml
vials (30 mg/vial) in a polyoxyethylated castor oil
(Cremophor EL®) 50% and dehydrated alcohol, USP (50%)
vehicle. The intact vials should be stored under
refrigeration and diluted prior to use. When diluted in
either 5% Dextrose Injection or 0.9% Sodium Chloride,
taxol concentrations of 0.3-1.2 mg/ml are physically and
chemically stable for at least 12 hours at room
temperature. (NCI Investigation Drugs; Pharmaceutical
Data (1990)). It has also been demonstrated that taxol
concentrations of 0.6 mg/ml diluted in either D5W or NS
and 1.2 mg/ml diluted in NS prepared in polyolefin
containers are stable for at least 25 hours at ambient
temperatures (20-23°C). (Waugh, et al. (1990) Am. J.
Hosp. Pharm. 48, 1520). Although these concentrations
have exhibited stability for the above periods of time,
they are not meant to limit the practice of the present
invention wherein any concentration of taxol may be
utilized.

All solutions of taxol exhibit a slight haziness
directly proportional to the concentrations of drug and
time elapsed after preparation. Formulation of a small
number of fibers in the solution (within acceptable limits
established by the USP Particulate Matter Test for LVP's)

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has been observed after preparation of taxol infusion
solutions. While particulate formation does not indicate
loss of drug potency, solutions exhibiting excessive
particulate matter formation should not be used.
Therefore, when administering via continuous infusion, in-
line filtration may be necessary and can be accomplished
by incorporating a hydrophilic, microporous filter with a
pore size no greater than 0.22 microns (IVEX-HP In Line
Filter Set-SL, 15", Abbott model # 4525 or equivalent)
into the fluid pathway distal to an infusion pump.

Taxol must be prepared in non-plasticized
solution containers (e.g., glass, polyolefin, or
polypropylene) due to leaching of diethylhexylphthlalate
(DEHP) plasticizer from polyvinyl chloride (PVC) bags and
intravenous tubing. Taxol must not be administered
through PVC intravenous or injection sets. Therefore,
polyolefin- or polyethylene-line sets, such as IV
nitroglycerin sets (or equivalent) should be used to
connect the bottle or bag (containing the taxol solution
for a continuous infusion) to the IV pump, a 0.22 micron
filter is then attached to the IV set, and then may be
directly attached to the patient's central access device .
If necessary, a polyolefin-line extension set (Polyfin™
Extension Set, MiniMed Technologies, Model #126) can be
used to provide additional distance between the IV pump
and the patient's central access device.

One category of taxol use would encompass the
prevention of recurrent stenosis (restenosis) post
therapeutic coronary- or peripheral-artery angioplasty or
atherectomy, after coronary bypass graft or stent surgery,
or after peripheral vascular surgery (e.g., carotid or
other peripheral vessel endarterectomy, vascular bypass,
stent or prosthetic graft procedure). A human dosing
schedule can consist of (but not be limited to) 24-hour
continuous IV pretreatment with up to about 0.5-2 mg/kg
(20-80 mg/m^2) prior to the vascular procedure, about 0.25-

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2 mg/kg (10-80 mg/m^2) continuous IV infusion over the 24
hours post-procedure, then about 0.25- 2 mg/kg (10-80
mg/m^2) continuous IV infusion over 24 hours every 21 days
for 1 to 6 cycles. Such a dosage is significantly lower
than that used to treat human cancers (approximately 4-6
mg/kg).

Another category of taxol use would encompass
the primary prevention, or the attenuation, of vascular
disease (atherosclerosis) development. Certain of these
applications (examples of which include the prevention of
cardiac allograft (transplant) atherosclerosis, the multi-
organ system failure resulting from the vascular
complications of diabetes mellitus or accelerated,
medically-refractory atherosclerosis in patients who are
poor surgical candidates) may require the later treatment
cycles to be continuous low-dose (1-5 mg/m^2/day) IV
infusions over 5-7 days. Each of the taxol treatments
will generally require pretreatment with dexamethasone 20
mg orally 14 and 7 hours prior to taxol, diphenhydramine
50 mg IV and cimetidine 300 mg IV 30 min prior to taxol,
to minimize potential episodes of allergic reaction.
Additional applications that may not be associated with a
surgical procedure include treatment of vascular
fibromuscular dysplasia, polyarteritis nodosa, and
Takayasu's arteritis. Each of the aforementioned
applications may also be amenable to selective, localized
application of sustained-release preparations of taxol (or
other microtubule-stabilizing agent) which would enable
high dosage local drug delivery with little systemic
toxicity.

Additionally, water soluble derivatives of taxol
can also be used in the present invention. The water
soluble derivatives of taxol, as described in U.S. Pat.
No. 5,157,049 to Haugwitz, et al. (incorporated herein by
reference) include, but are not limited to, 2'-succinyl-
taxol; 2'-succinyl-taxol triethanolamine; 2'-glutaryl-

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taxol; 2'-glutaryl-taxol triethanolamine salt; 2'-O-ester
with N-(dimethylaminoethyl) glutamide; 2'-O-ester with N-
(dimethylaminoethyl) glutamide hydrochloride salt. These
water soluble taxol derivatives can be administered in a
dosage schedule analogous to that given above for taxol
with the appropriate modifications pending clarification
of the pharmacokinetics of these agents.

A pharmaceutical composition comprising an
effective amount of water soluble derivative of taxol as
an active ingredient is easily prepared by standard
procedures well known in the art, with pharmaceutically
acceptable non-toxic sterile carriers, if necessary. Such
preparations could be administered orally or in injectable
form, or directly to an affected area, to a patient at
risk of developing or suffering from atherosclerosis to
prevent or reduce the development of the disease.

The following examples illustrate the
effectiveness of taxol (or other microtubule-stabilizing
agents including, but not limited to, water soluble
derivatives of taxol) in inhibiting the proliferation and
migration of vascular smooth muscle cells, and should not
be used to limit the scope of the present invention.

Example 1

The in vitro ability of cultured VSMCs,
pretreated with different taxol concentrations, to invade
filters coated with reconstituted basement membrane
proteins was tested to evaluate how taxol-induced
microtubule bundling would impair cell processes necessary
for in vivo neointimal formation.

Vascular Smooth Muscle Cells (VSMCs) were
isolated by collagenase/elastase enzymatic digestion of
the medial layers of the rat aorta obtained from 6 month
old Wistar rats. The cells were maintained in culture
with 10% fetal calf serum, high glucose DMEM, and amino
acid supplement. Cell cultures were maintained at 37°C in
5% CO_2.

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After 18-hour taxol pre-treatment in culture,
cells were fixed in 3.7% formalin, permeabilized with 1 %
Triton X-100, and polymerized tubulin was labelled with
mouse anti-ß-tubulin antibody (SMI 62 monoclonal antibody
to polymerized ß-tubulin, Paragon Biotec, Inc., Baltimore,
MD). Secondary labelling was achieved with silver-
enhanced, 1 nm gold-conjugated rabbit anti-mouse antibody
(Goldmark Biologicals, Phillipsburg, NJ). Representative
light photomicrographs from (A) control, (B) 0.1 nM taxol,
(C) 1 nM taxol, and (D) 10 nM taxol treated VSMCs are
shown in Figure 4.

Chemonivasion (Boyden chamber) assays were
performed using modified Boyden chamber (Albini, et al.
(1987) Cancer Res., 47:3239-3245), comprising an upper
chamber separated from a lower chamber by a porous PVPF
filter. PVPF filters (8 μm pore diameter, Nucleopore
Filters, Pleasonton, CA) were coated and air dried
consecutively with solutions containing 100 μg/ml type I
collagen, 5 μg/ml fibronectin, and 5 μg reconstituted
basement membrane (produced from the Englebreth-Holm-Swarm
tumor (Kleinman, et al. (1986) Biochemistry, 25:312-318),
producing a continuous 10 μm thick coating of matrix
material. Boyden chambers were assembled by adding 10
ng/ml PDGF BB in DMEM to the lower (chemoattractant)
chamber. Cells (approximately 200,000) suspended in DMEM
containing 0.1% BSA were then added to the upper chamber.
Some of the cells used in this assays were pretreated 18
hours with taxol (concentration 30 pM to 100 nM) in
culture. In the taxol-treated groups, taxol was added to
the upper and lower chambers at the same concentration as
that used for pretreatment. The chambers were then
incubated for 4 hours at 37°C in a 5% CO_2 atmosphere. At
the end of the incubation period, the cells were fixed and
stained with hematoxylin and eosin. The cells on the
upper surface (non-invaders) were mechanically removed and
the cells on the underside of the filter (invaders) were

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counted under 400 x magnification (four random fields were
counted per filter and all experiments were run in
triplicate, and each triplicate assay was repeated at
least three times on separate occasions using different
VSMC preparations). Chemotaxis was assayed in analogous
fashion in the Boyden chambers described above, except
that the reconstituted basement membrane was omitted.
This chemoinvasion assay is accepted by those skilled in
the art as exhibiting high correlation between
invasiveness in vitro and cellular behavior as it occurs
in vivo (Iwamoto, Y., et al. (1992) Advances In
Experimental Medicine & Biology, 324:141-9).

Using the PDGF-BB as an attractant, taxol
inhibited VSMC invasion with half-maximal inhibitory
concentration of 0.5 nM. Taxol caused essentially
complete inhibition at 100 nM, and significant inhibition
was still resolvable at 30 pM (the lowest dose
used)(Figure 1). A chemotaxis assay (filter coated only
with fibronectin and collagen I, without basement membrane
proteins occluding the filter pores) with PDGF-BB as the
attractant was performed in analogous fashion, yielding
the identical outcome. These results demonstrate that
taxol, at least at nanomolar drug levels, inhibits VSMC
invasion primarily via inhibition of locomotion and/or
shape changes, rather than by inhibiting cellular
secretion of collagenases and metalloproteinases, which
are known to be necessary for VSMC to penetrate basement
membrane proteins in this assay.

Gelatinase zymography was performed on the
supernatants removed after the 4 hour conclusion of the
Boyden assays described above. Gelatin-degrading
proteinases secreted into the media by VSMCs were analyzed
by non-reducing sodium dodecyl sulfate-polyacrylamide gel
electrophoresis in 10% polyacrylamide gels containing 0.1%
(w/v) gelatin. Following electrophoresis, the gelatinases
were renatured by incubating the gel for 30 min. at 23°C

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in 2.5% (v/v) Triton X-100 followed by 18 hour incubation
at 37°C in 0.2 M NaCl, 5 mM $CaCl_2$ 0.02% Brij 35 (w/v), 50
mM Tris-HCI (pH 7.6). The gels were stained for 90
minutes with 0.5% Coomassie Brilliant Blue G-250 and
destained with 10% acetic acid, 40% methanol.
Gelatinolytic activity was indicated by a clear band
against the background of blue-stained gelatin.

These gelatinase zymography assays from the
Boyden chamber invasion experiments confirm that the level
of VSMC collagenase secretion did not vary significantly
over the taxol range 30 pM to 100 nM, compared to control
(Figure 2, inset).

Example 2

To confirm the fact that microtubule
stabilization and hyperpolymerization is the critical and
sufficient factor involved in taxol-inhibition of VSMC
invasiveness, the chemoinvasion (Boyden chamber) assay was
run with deuterium oxide (^2H_2O, heavy water). Deuterium
oxide enhances microtubule/tubulin polymerization via a
mechanism distinct from that of taxol. A combination of
the isotope and solvent effects of deuterium oxide
reversibly increases microtubule polymerization both by
reducing the critical concentration for polymerization for
$\alpha\beta$-tubulin heterodimers via enhanced tubulin hydrophobic
interactions (Itoh, T.J., et al. (1984) Biochim. Biophys.
Acta., 800:21-27), and by converting a population of
unpolymerizable tubulin to the polymerizable form
(Takahashi, T.C., et al. (1984) Cell Struct. Funct., 9:45-
52).

VSMC's were isolated by collagense/elastase
enzymatic digestion of the medial layers of the rat aorta
obtained from 6 month old Wistar rats. The cells were
maintained in culture with 10 % fetal calf serum, high
glucose DMEM, and amino acid supplement. Cell cultures
were maintained at 37°C in 5% CO_2.

In deuterium oxide-treated cells, ^2H_2O (v/v) was

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substituted for water (H$_2$O) in the preparation of the DMEM
from concentrated stock. After 18-hour deuterium oxide
pre-treatment in culture, cells were fixed in 3.7%
formalin, permeabilized with 1 % Triton X-100, and
polymerized tubulin was labelled with mouse anti-δ-tubulin
antibody (SMI 62 monoclonal antibody to polymerized β-
tubulin, Paragon Biotec, Inc., Baltimore, MD). Secondary
labelling was achieved with silver-enhanced, 1 nm gold-
conjugated rabbit anti-mouse antibody (Goldmark
Biologicals, Phillipsburg, NJ). Representative light
photomicrographs from (A) control, and (B) 75% deuterium
oxide treated VSMCs are shown in Figure 5.

Chemoinvasion assays were performed using a
modified Boyden chamber, consisting of an upper chamber
separated from a lower chamber by a porous PVPF filter.
PVPF filters (8 μm pore diameter, Nucleopore Filters,
Pleasonton, CA) were coated and air dried consecutively
with solutions containing 100 μg/ml type I collagen, 5
μg/ml fibronectin, and 5 μg reconstituted basement
membrane (produced from the Englebreth-Holm-Swarm tumor),
producing a continuous 10 μm thick coating of matrix
material. Boyden chambers were assembled with PDGF-BB 10
ng/ml in DMEM in the lower (chemoattractant) chamber, then
cells (approximately 200,000) suspended in DMEM containing
0.1% BSA were added to the upper chamber. Some of the
cells used in these assays were pretreated 18 hours with
deuterium oxide (25%, 50%, or 75% v/v substitution for
H$_2$O) in culture. In the deuterium oxide-treated groups,
^{2}H$_2$O substituted DMEM (v/v) was added to the upper and
lower chambers at the same concentration as that used for
pretreatment. The chambers were then incubated for 4
hours at 37°C in a humidified 5% CO$_2$ atmosphere. At the
conclusion of the experiment, the filters were removed and
the cells were fixed and stained with hematoxylin and
eosin. After the cells on the upper surface of the filter
(non-invaders) were mechanically removed, the cells on the

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underside (invaders) were counted under 400 X
magnification (four random fields were counted per filter
and all experiments were run in triplicate).

Pretreating cultured VSMCs for 18 hours with
25%, 50% or 75% deuterium oxide caused dose-dependent
microtubule hyperpolymerization similar to that observed
with taxol. This treatment likewise inhibited PDGF-
mediated VSMC Boyden chamber invasion in a dose-dependent
fashion, achieving half-maximal inhibition at 25% ^2H_2O, and
nearly complete inhibition at 75% ^2H_2O (Figure 3).

Example 3

In addition to cell recruitment and migration,
the various growth regulatory molecules elaborated after
arterial injury, such as PDGF and bFGF, are also
implicated in mitogenesis and cellular proliferation. To
measure the effect of taxol on VSMC DNA synthesis,
[^3H] thymidine incorporation was measured. VSMCs were
plated at 4.5×10^4 on 24-well plates. Following 5 hr.
incubation in 10% FCS+DMEM, 0.5 mCi [^3H] thymidine was
added and the incubation continued for an additional 16
hrs. Cells were washed twice with phosphate-buffered
saline, extracted with 10% TCA for 2 hrs. on ice, then
centrifuged at 2,000 g for 10 mins. Supernatants were
decanted and pellets were solubilized in 0.5 ml of 1 N
NaOH. After neutralizing with 0.5 ml of 1 N HCl,
[^3H] thymidine uptake was determined by a Beckman liquid
scintillation counter. VSMCs were treated with the
various concentrations of taxol for both the 18 hr. prior
to the addition of the thymidine and during thymidine
incorporation. Each condition of these experiments was
performed in triplicate.

Taxol inhibited cultured VSMC [^3H] thymidine
incorporation, an index of cell division, in a dose-
dependent fashion, with a half-maximal inhibitory
concentration of 5 nM. Taxol caused essentially complete
inhibition at 100 nM, and significant inhibition was

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resolvable at 1 nM (Figure 1). That this inhibitory
profile differs somewhat from that of invasion and
chemotaxis, demonstrating one log-concentration-unit lower
sensitivity but with steeper concentration-dependence,
likely arises because of the considerably different roles
played by mircotubules between these processes. Taxol
also inhibited PDGF-BB-stimulated c-fos mRNA expression in
this cultured VSMC model, in a dose-dependent fashion,
with a half-maximal inhibitory concentration of 1 nM, with
essentially complete inhibition above 20 nM. Thus,
inhibition of immediate early gene induction is another
important mechanism by which taxol blocks growth factor
stimulation in VSMCs, and may underlie, at least in part,
the thymidine incorporation results.

Thus, taxol significantly inhibits cultured VSMC
in vitro invasion and proliferation through interference
with microtubule function, disrupting locomotion and the
ability to alter shape, as well as growth-factor
stimulated early gene expression and cell proliferation,
at concentrations one hundred - to one thousand-fold lower
than used to treat human cancer.

Example 4

Incorporation of the thymidine analog,
bromodeoxyuridine (BrDU) was measured to determine the
effect of deuterium oxide on VSMC DNA synthesis. VSMCs
were plated at 4.5×10^4 on 24-well plates. Following 20
hr incubation in 10% FCS+DMEM at various ^2H_2O
concentrations, 10 μM BrDU was added and the incubation
continued for an additional 4 hr. Cells were washed twice
with phosphate-buffered saline (PBS) and fixed with 100%
methanol (-20°C) for 10 minutes. The cells were incubated
for 2 hr with 1N HCl to denature the DNA, and subsequently
washed 4 times in PBS. Mouse monoclonal BrDU antibody
(Boehringer Mannheim) in 2% BSA-PBS was incubated with
cells for 1 hr. After PBS wash, goat anti-mouse antibody
conjugated with alkaline phosphatase was added. Cell

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nuclei containing BrDU substituted for thymidine stained
red with alkaline phosphatase substrate, while all other
nuclei stained blue. The fraction of BrDU-positive nuclei
was compared between control (defined as 100%) and that of
the deuterium oxide-pretreated groups.

5   The results indicated that deuterium oxide,
similar to taxol, inhibited cultured VSMC proliferation
and DNA synthesis in a dose-dependent fashion, consistent
with the critical balance of microtubule-tubulin dynamics
in VSMC proliferation.

10   While taxol and deuterium oxide potentially have
multiple intracellular effects, the coincidence of their
parallel effects on microtubules (despite different
mechanisms of action) and on VSMC functionality at
multiple levels, indicates that the common microtubule

15   stabilizing mechanism of action is responsible for the
observed functional changes. Thus, based on the results
of experiments with both taxol and deuterium oxide, it is
evident that microtubules are involved in the control of
the most critical and sensitive intracellular mechanisms

20   necessary for VSMCs to undergo the multiple
transformations involved in the development of
atherosclerosis and restenosis after arterial injury,
making microtubules particularly strategic targets to
influence the outcome.

25   Example 5
              Under a protocol approved by the National
Institute on Aging Animal Care and use Committee, 6 month
Wistar rats from the GRC colony were anesthetized with 20
mg/kg body weight pentobarbital, 2 mg/kg body weight

30   ketamine, and 4 mg/kg body weight xylazine
intraperitoneally. The left external carotid artery was
cannulated with 2-French Fogarty embolectomy catheter,
inflated with saline and passed three times up and down
the common carotid artery to produce a distending,

35   deendothelializing injury. The animals were treated with

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2 mg/kg body weight taxol solution or the control animals
with vehicle alone (13.4 ml/kg body weight per day of
1:2:2:165 DMSO:Cremophor EL:Dehydrated ethanol:phosphate
buffered saline) by intraperitoneal injection beginning 2
hours after injury. The taxol solution or vehicle alone
was administered once daily, as an intraperitoneal
injection, for the next 4 days. After 11 days the animals
(8 taxol-treated and 10 vehicle-treated) were anesthetized
as above and the carotid artery was isolated and fixed in
10% buffered formalin and embedded in paraffin. Cross
sections of the carotids were mounted on microscope slides
and stained with hematoxylin and eosin stain. The image
of the carotid artery was projected onto a digitizing
board and the cross sectional areas of the intima and the
media were measured. The results are shown in Figure 2.
As indicated in the prior art (Ferns, G.A.A. et al. (1991)
Science, 253:1129-1132) the rat carotid artery injury
model of restenosis can be useful in the study of human
restenosis, and indicate potential therapeutic action in
humans.

Quantitative analysis of injured carotid
segments showed that taxol treatment reduced the
neointimal area by 70% compared to vehicle treated animals
(Table I) (*P < 0.001; †P=NS; ‡P < 0.001). Several of the
taxol-treated animals showed virtually no discernable
neointima (in the presence of denuded endothelium, proving
injury), while all vehicle treated animals demonstrated at
least modest neointimal thickening.

While the in vivo systemic taxol dose used in
these experiments (2 mg/kg) is significantly lower than
that ordinarily used to treat human cancers (approximately
3-6 mg/kg), dramatically lower systemic dosing with
sustained or even improved efficacy could be possible
combining a pretreatment regimen with the optimal
treatment duration. Furthermore, since the goal of
therapy is to keep the "activated" VSMCs in check, or

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preferably to prevent activation in the first place, until
the stimulus for growth and migration has resolved (rather
than causing cytotoxicity resulting in cell death), the
goal of short-term therapy with limited toxicity may be
possible in humans. Ultimately, local sustained-release
delivery systems may offer the best solution to prevent
restenosis post-angioplasty, enabling high local
concentrations of drug delivery and essentially
eliminating problems of systemic toxicity. Drug delivery
systems that can be valuable include drug-impregnated
polymer-coated metallic stents, biodegradable drug-eluting
polymer stents, and genetically primed endothelial cells
to coat metallic stents or be delivered directly as a
local endothelial cell covering. (Muller, D.W.M. et al.
(1991) JACC 17:126b-131b). These systems allow safe use
of a chemotherapeutic agent without systemic side effects.
Alternatively, treatment may involve a period of
pretreatment (i.e., before vascular surgery) via
continuous intravenous infusion for a period of time,
followed by a different therapy during (local, direct
delivery) or after (oral, injection) surgery.

         The above examples teach taxol's (or other
microtubule-stabilizing agent including, but not limited
to, water soluble derivatives of taxol) potential
beneficial uses to prevent artery blockage and thereby
reduce the possibility of, or prevent, heart attacks,
strokes, kidney failure and renal dialysis, blindness,
limb amputations, nerve loss, need for corrective vascular
surgery/angioplasty or organ transplantation, and
premature and permanent disability requiring chronic
hospitalization. The invention has been described in
detail, but it will be understood that the invention is
capable of other different embodiments. As is readily
apparent to those skilled in the art, variations and
modifications can be affected within the spirit and scope
of the invention. Accordingly, the foregoing disclosure

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and description are for illustrative purposes only, and do not in any way limit the invention, which is defined only by the claims.

TABLE 1

Group	Intima (mm^2)	Media (mm^2)	I/M
Vehicle	0.09±0.01	0.14±0.01	0.66±.08
Taxol	0.03±0.01[*]	0.16±0.02[†]	0.18±.04[‡]

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We claim:

1.   A method of preventing or reducing an arterial disease in a patient comprising:

treating said patient with a pharmaceutical preparation comprising a therapeutically effective amount of a microtubule stabilizing chemotherapeutic agent.

2.   The method of claim 1 wherein said microtubule stabilizing chemotherapeutic agent is taxol or a water soluble taxol derivative.

3.   The method of claim 2 wherein said water soluble taxol derivative is selected from the group consisting of a 2'-succinyl-taxol; 2'-succinyl-taxol triethanolamine; 2'-glutaryl-taxol; 2'-glutaryl-taxol triethanolamine salt; 2'-O-ester with N-(dimethylaminoethyl) glutamide; and 2'-O-ester with N-(dimethylaminoethyl) glutamide hydrochloride salt.

4.   The method of claim 1 wherein said arterial disease is atherosclerosis.

5.   The method of claim 2 wherein said arterial disease is atherosclerosis.

6.   The method of claim 3 wherein said arterial disease is atherosclerosis.

7.   The method of claim 1 wherein said arterial disease is restenosis.

8.   The method of claim 2 wherein said arterial disease is restenosis.

9.   The method of claim 3 wherein said arterial disease is restenosis.

10. The method of claim 1 wherein said treating step is comprised of systemic delivery of said pharmaceutical preparation.

11. The method of claim 1 wherein said treating step is comprised of local delivery of said pharmaceutical preparation.

12. A drug delivery system comprising a means for locally delivering a microtubule stabilizing

chemotherapeutic agent in a therapeutically effective
amount to reduce or prevent the development of a
neointimal fibroproliferative lesion.

    13. The drug delivery system of claim 12
wherein said microtubule stabilizing chemotherapeutic
agent is comprised of taxol or a water soluble taxol
derivative.

    14. A use of a microtubule stabilizing
chemotherapeutic agent in a pharmaceutical composition in
the prevention or reduction of arterial disease in a
patent.

    15. The use of claim 14 wherein said
microtubule stabilizing chemotherapeutic agent is taxol or
a water soluble taxol derivative.

    16. The use of claim 15 wherein said water
soluble taxol derivative is selected from the group
consisting of a 2'-succinyl-taxol; 2'-succinyl-taxol
triethanolamine; 2'-glutaryl-taxol; 2'-glutaryl-taxol
triethanolamine salt; 2'-O-ester with N-
(dimethylaminoethyl) glutamide; and 2'-O-ester with N-
(dimethylaminoethyl) glutamide hydrochloride salt.

    17. The use of claim 14 wherein said arterial
disease is atherosclerosis.

    18. The use of claim 15 wherein said arterial
disease is atherosclerosis.

    19. The use of claim 16 wherein said arterial
disease is atherosclerosis.

    20. The use of claim 14 wherein said arterial
disease is restenosis.

    21. The use of claim 15 wherein said arterial
disease is restenosis.

    22. The use of claim 16 wherein said arterial
disease is restenosis.

    23. The use of claim 14 wherein said treating
step is comprised of systemic delivery of said
pharmaceutical preparation.

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24.   The use of claim 14 wherein said treating
step is comprised of local delivery of said pharmaceutical
preparation.

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Figure 1
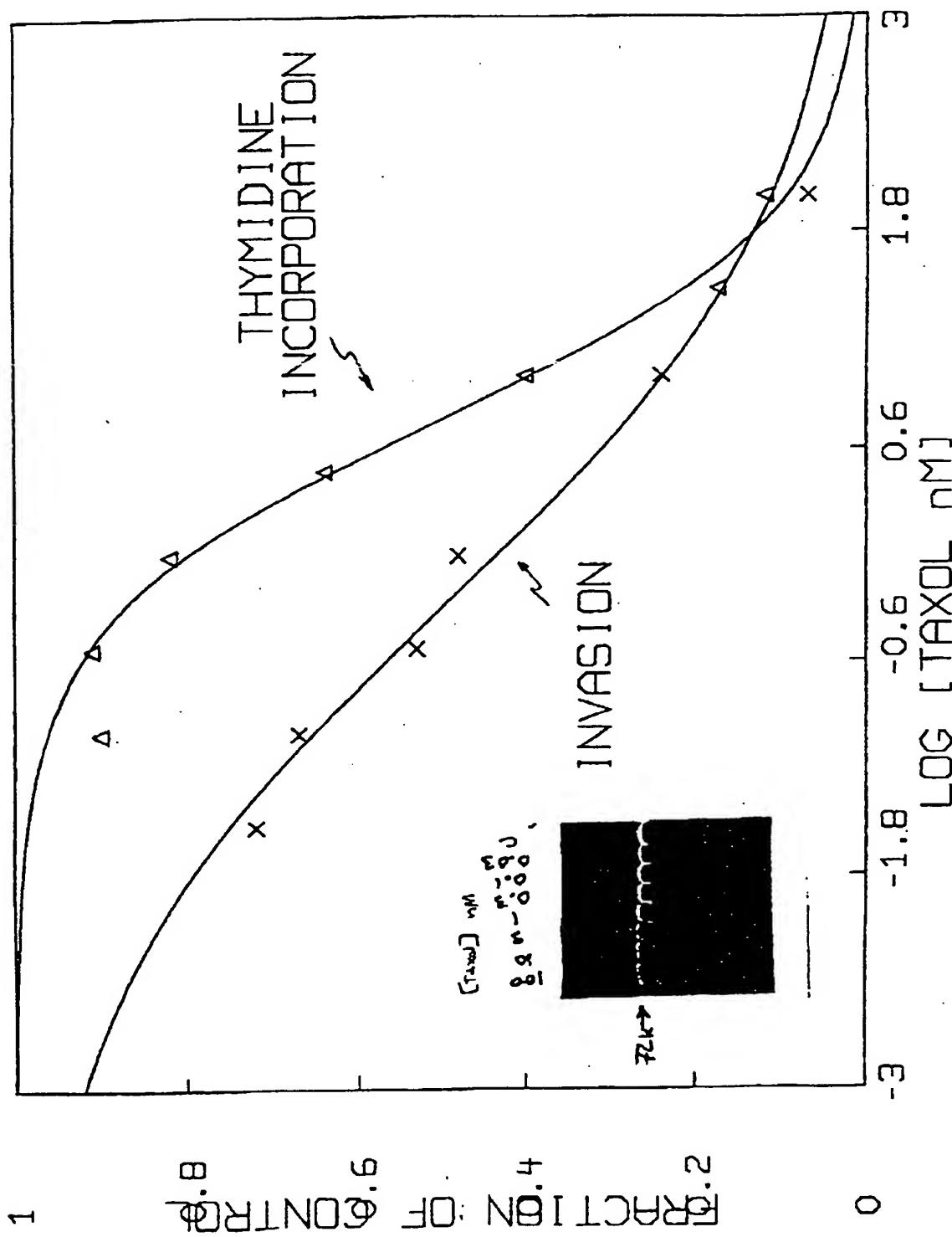
Figure 2
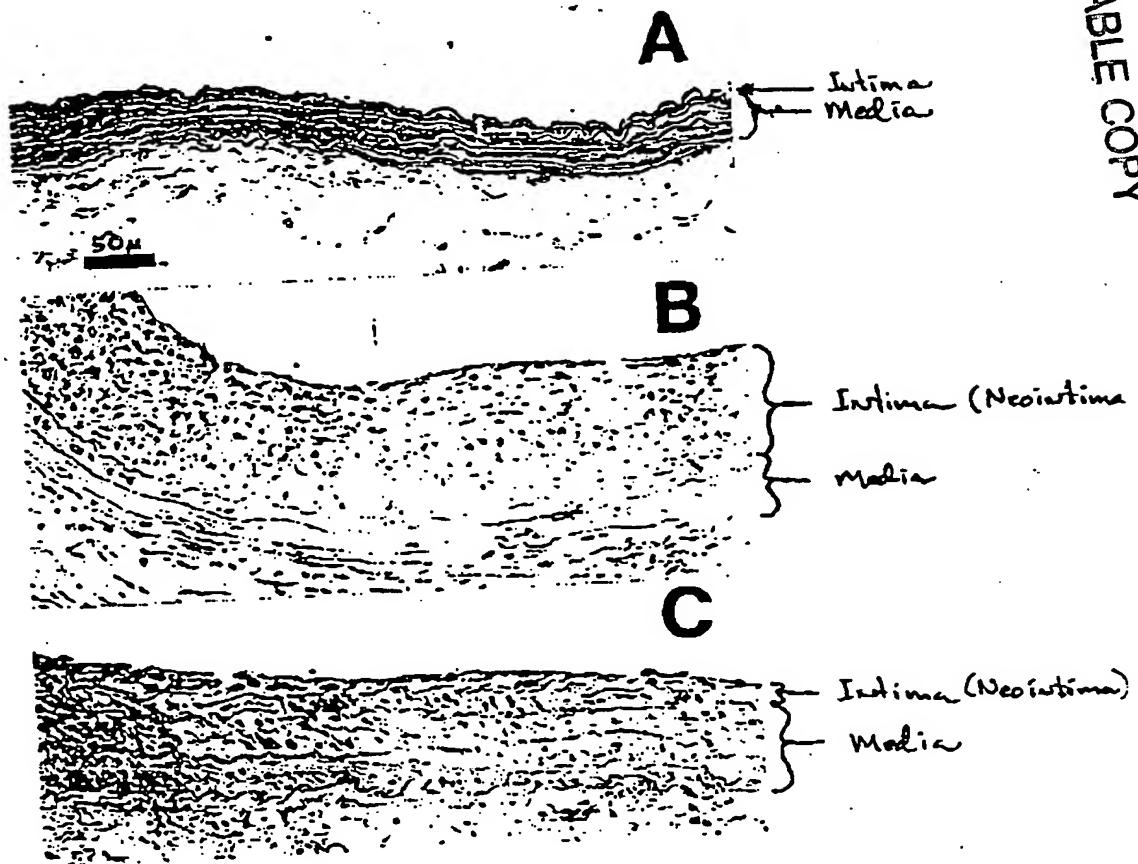
TAXOL INHIBITS THE ACCUMULATION OF INTIMAL SMOOTH MUSCLE
CELLS 11 DAYS AFTER BALLOON CATHETER INJURY OF RAT CAROTID ARTERY.
HEMATOXYLIN- AND EOSIN-STAINED CROSS SECTIONS OF BALLOON-
CATHETERIZED RAT COMMON CAROTID ARTERIES 11 DAYS POST-PROCEDURE.
(A) UNINJURED ; (B) INJURED + VEHICLE ; (C) INJURED + TAXOL

Figure 3   3/5

VSMC Invasion and BrDU Incorportion:
Effect of Deuterium Oxide
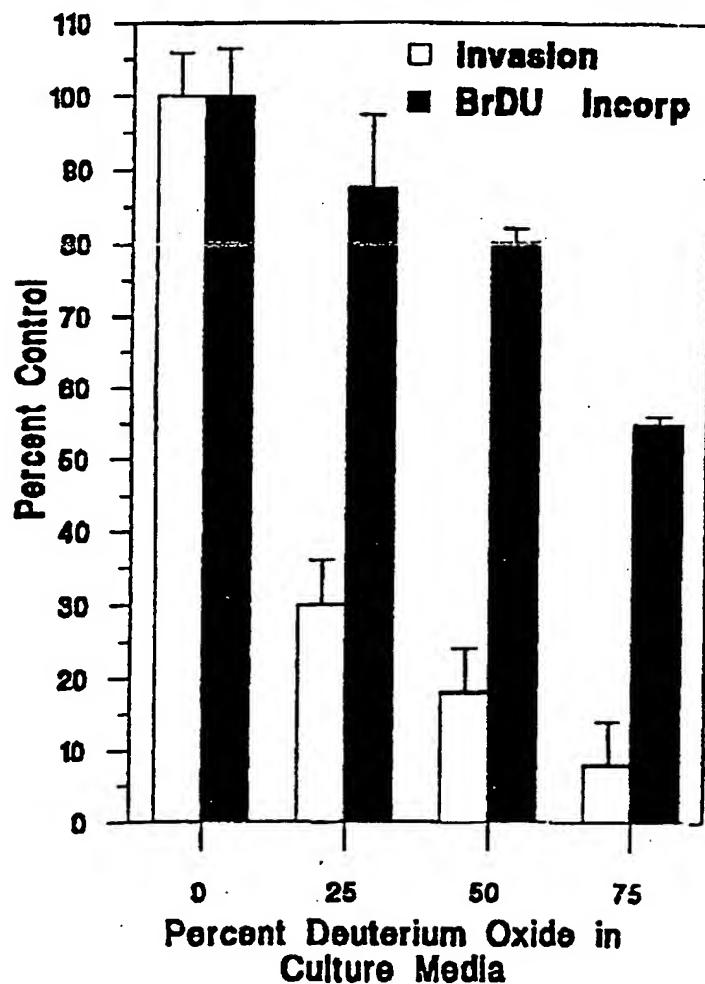
Figure 4
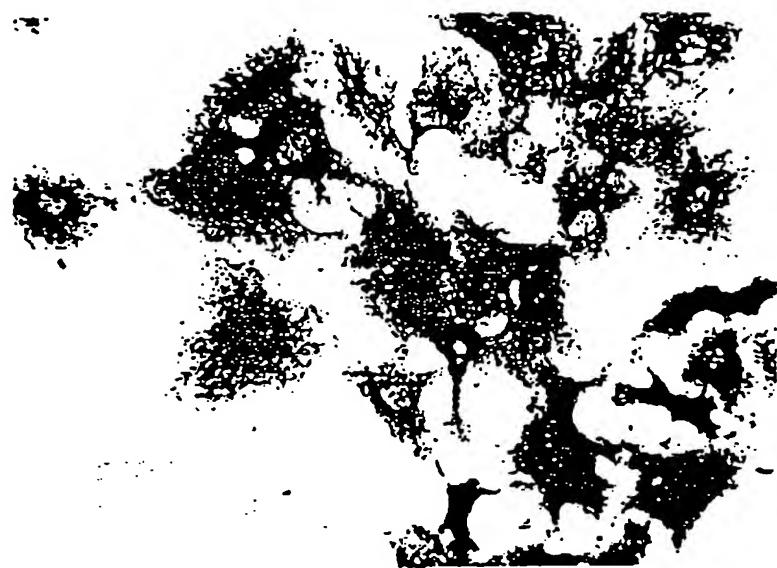
Figure 5.

B. 75% DEUTERIUM OXIDE

A. CONTROL

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K31/335   A61K31/54   A61K31/535   A61K31/495   A61K31/445
A61K31/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ATHEROSCLEROSIS vol. 44 , 1982 pages 385 - 90 CHALDAKOV G. N. 'Antitubulins- A New Therapeutic Approach for Atherosclerosis?' see the whole document	1,4,7, 10-12, 14,17, 20,23,24
Y	see the whole document	2,3,5,6, 8,9,13, 15,16, 18,19, 21,22
Y	US,A,5 157 049 (HAUGWITZ ET AL) 20 October 1992 cited in the application see the whole document	1-11, 14-24
X	see the whole document	12,13

-/--

X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 1994	1 4. 12 94

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	METH. AND FIND. CLIN. PHARMACOL. vol. 7, no. 4 , 1985 pages 183 - 7 BARD ET AL 'Normolipemic Activities of Acrylophenone Derivatives with Antimicrotubular Properties' see page 186	1,4,7, 10-12, 14,17, 20,23,24
Y	see the whole document	2,3,5,6, 8,9,13, 15,16, 18,19, 21,22
X	CIRCULATION vol. 80/S-II , 1989 page 66 CURRIER ET AL 'Colchicine Inhibits Restenosis After Iliac Angioplasty in the Atherosclerotic Rabbit' cited in the application no.0283 see the whole document	1,4,7, 10-12, 14,17, 20,23,24
Y	see the whole document	2,3,5,6, 8,9,13, 15,16, 18,19, 21,22
Y	J. OF INT. MED. vol. 233 , 1993 pages 215 - 26 LöVQUIST ET AL 'Pathophysiological Mechanism for Restenosis Following Coronary Angioplasty: Possible Preventive Alternatives' see page 221, column 2, paragraph 2	1-11, 14-24
X	'The Merck Index' 1989 , MERCK CO. INC. no.9049 see page 1435	12,13

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PCT/US 94/08578

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		AU-B-	620605	20-02-92
		AU-A-	3286189	05-10-89
		CA-A-	1330436	28-06-94
		DE-D-	68917129	01-09-94
		EP-A-	0406274	09-01-91
		IL-A-	89503	27-02-94
		JP-T-	3505725	12-12-91
		WO-A-	8908453	21-09-89